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# Investigating the Antidiabetic Effect of Metformin on Pyruvate Carboxylase

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# Investigating the Antidiabetic Effect of Metformin on Pyruvate Carboxylase

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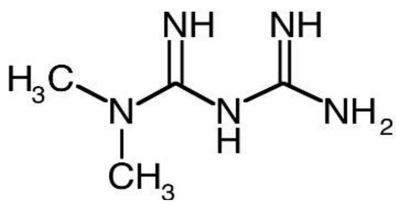
**Abstract**: Metformin is the most commonly prescribed treatment for type II diabetes. While the clinical effects of this generic drug are established, exact targets of metformin action remain unclear. The hypothesis of the present study was that metformin lowers blood glucose through inhibition of pyruvate carboxylase (PC), an enzyme catalyzing the first committed step of gluconeogenesis. Because metformin is relatively low cost, it is widely accessible to communities worldwide; currently, 150 million individuals depend on metformin for treatment. Thorough investigation of all targets involved in action of metformin becomes crucial, as it further explains the adverse effects of the drug. The specific activity of purified bovine PC was measured spectrophotometrically through the loss of absorbance at 340nm, following the oxidation of NADH. The design involved a series of enzyme-coupled assays treated with or without metformin (250-2,500 μM). Waller-Duncan post-hoc analysis revealed significant inhibition between controlled and treated groups at 500-2,500μM of metformin for the assays. Supporting results provide further insight into mechanisms of action behind metformin, as well as provide further explanation for the effects users experience.

**Keywords**: *gluconeogenesis, metformin, pyruvate carboxylase, type II diabetes, lactic acidosis*

Type II diabetes affects communities worldwide. As of 2014, 29.1 million people were diagnosed with diabetes in the United States alone (Centers for Disease Control and Prevention, 2014a). Metformin lowers blood glucose levels and promotes insulin sensitivity of target organs. While gluconeogenesis has been established as the key pathway through which metformin mediates its physiological effects (Gastaldelli et al., 2000; Magnusson, Rothman, Katz, & Shulman, 1992; Rossetti et al., 1993), the exact targets of the drug are currently unknown, although there have been several proposed candidates (e.g., Cao et al., 2014; Foretz et al., 2010; Madiraju et al., 2014; Shaw et al., 2005; Zhou et al., 2001). Past data suggests metformin might induce its anti-diabetic effects through the inhibition of pyruvate carboxylase (PC), a primary enzyme involved in the first committed step of gluconeogenesis. Thus, the hypothesis of this study is that metformin inhibits gluconeogenesis through the inhibition of pyruvate carboxylase at concentrations near pharmacokinetic range. Analysis of the effects of metformin on PC will help determine if the drug directly targets PC enzymatic activity. Support of this hypothesis will suggest a new target involved in the anti-gluconeogenic action of metformin. Currently, the most current [drug] therapies available for treatment of type II diabetes fall under 11 chemical groups that include the guanidines (Close et al., 2012). Derived from the plant *Galega officinalis*, uses for guanidines in treating diabetes were documented as early as the *17th* century, when physicians first began to describe and characterize type II diabetes (Bailey & Day, 2004). It was not until 1918 that Watanabe first published data demonstrating the guanidine plant extract possessed hypoglycemic activity. In 1994 the Federal Drug Administration in the United States approved dimethylbiguanide hydrochloride (Figure 1), or Metformin; itis currently the most commonly prescribed drug for type II diabetes treatment (Bailey & Day, 2004; He & Wondisford, 2015). Given clinically in Europe since the 1920's (Kaneto et al., 2016), Metformin is prescribed for treatment of hyperglycemia, a primary characteristic of type II diabetes (Irons & Minze, 2014).

*Figure 1.* Carbon structure of Metformin, or dimethylbiguandine. Adapted from "Glyburide Microionized and Metformin Hydrochloride," by E.M. Lamos, S.A. Stein, and S.N. Davis, 2012, *Expert Opinion on Pharmacotherapy.*



**Purpose and Significance**

Given the many contradictory findings, this study aimed to compare the effects of metformin on PC. Specific ranges of concentrations (250-2,500μM) were used to assess past observations that proposed other mechanisms behind metformin action (Cao et al., 2014; Madiraju et al., 2014; Foretz et al., 2010; Zhou et al., 2001). Support of this hypothesis will aid in supporting PC as one of the key targets involved in the route of action for metformin. Observed inhibition will also support the metabolic control theory, stating a major metabolic pathway such as gluconeogenesis cannot solely be controlled by one primary enzyme (Fell, 1997).

**Adverse Effects of Metformin**

The importance of perusing metformin is also highlighted by the documented adverse effects of the drug. Gastrointestinal imbalances such as increased flatulence, diarrhea, abdominal pain, and decreased absorption of vitamin B12 have been reported (He et al., 2009). Metformin users with predisposing conditions, such as liver or kidney disease, fall risk for developing lactic acidosis: a toxic and potentially deadly condition with severe symptoms such as vomiting, diarrhea and renal impairment (Duong et al., 2013). It is of benefit to fully understand metformin, as the drug is widely used around the world for a disease that is quickly becoming a pandemic.

**Type II Diabetes Prevalence in Underrepresented Populations**

With the incidence of type II diabetes growing at astonishing rates, 2 out of 5 people in the U.S. are expected to develop type II diabetes in their lifetimes (Centers for Disease Control and Prevention, 2014a). More than half of non-Hispanic black women and Hispanic men and women are predicted to develop the disease within their lifetime (Centers for Disease Control and Prevention, 2014b). The observations from this study helped provide greater insight into the specific targets involved in diabetic treatment that help aid in the development of more effective methods for treatment that will provide efficient care to these growing and underrepresented populations.

## LITERATURE REVIEW

**Type II Diabetes**

The disease state of type II diabetes is characterized by an increase in fasting glucose production and increased insulin resistance. The physiological effects of type II diabetes are mediated via many metabolic pathways including gluconeogenesis, which produces glucose from non-carbohydrate sources when stressed or starving (Burgess et al., 2007; Cox & Nelson, 2013). The sources for gluconeogenesis, lactate and pyruvate, are derived from the breakdown of fat and protein stores that the body utilizes throughout these periods (Hanson & Owen, 2013). In the case of type II diabetes, gluconeogenesis is inappropriately upregulated in the liver, triggered by abnormal drops in insulin levels (Hanson & Owen, 2013). Insulin is secreted by pancreatic β islet cells, which critically function to control and stabilize blood glucose levels (Guerra et al., 2005). During high carbohydrate states in a non-diabetic patient, the body responds through immediate secretion of insulin and innate suppression of gluconeogenesis. However, cells become resistant to the effects of insulin in type II diabetes, leading to decreased glucose stability and persistent hyperglycemic conditions even during fasting states (Hanes & Krishna, 2010). Regulation of hepatic gluconeogenesis during hyperglycemic states has therefore been identified as a key element involved in the route of action for metformin (An & He, 2016).

**Phosphoenolpyruvate Carboxykinase.**

Hepatic gluconeogenesis involves two main enzymes, PC in the mitochondria and phosphoenolpyruvate carboxykinase (PEPCK) in the cytosol. PEPCK catalyzes the last committed step of gluconeogenesis and is responsible for the production of phosphoenolpyruvate from oxaloacetate, see Figure 2; (Hanson et al., 2013; Hanson & Garber, 1972). Rognstad (1979) established PEPCK as dominant over control of gluconeogenesis due to its rate limiting action over the pathway. Expression of PEPCK increases 2.9-fold in mouse adipose and liver tissue during many forms of chemically induced diabetes (Veneziale, Donofrio, & Nishimura, 1983) suggesting a role in diabetic pathogenesis and maintenance. PEPCK's dominant role in the reaction has even allowed it to be used in molecular and pharmaceutical studies as a reliable indicator of gluconeogenic activity (Chakravarty et al., 2005). Yet other studies have argued against the close relation of PEPCK and control of gluconeogenesis during type II diabetes. In high-fat-fed diabetic hyperglycemic mice, no significant correlation between PEPCK mRNA expression and high plasma glucose concentrations were found (Samuel et al., 2009). *In vitro* protein analysis of isolated murine hepatic cells suggested a weak correlation between PEPCK protein levels and diabetic hyperglycemia. The study generated a metabolic control coefficient of 0.18 for PEPCK, suggesting weak control over gluconeogenesis (Burgess et al., 2007). The aforementioned data challenges the notion that PEPCK is dominant in the control of gluconeogenesis, leaving room for other possible candidates of control.

### Pyruvate Carboxylase

PC is an anapleurotic enzyme that catalyzes the first committed step of gluconeogenesis (Jitrapakdee et al., 2008) and converts pyruvate into oxaloacetate (see Figure 2). There is renewed interest in PC as data suggests greater involvement for control of gluconeogenesis during type II diabetes.

During the disease state, insulin released from pancreatic β-islet cells becomes desensitized, leading to increased insulin resistance in patients even during hyper states of insulin secretion (Guerra et al., 2005). Proper receptor signaling for glucose uptake by target organs fails, leading to constant hyperglycemic conditions in circulation (Liang et al., 2011). Researchers have found high levels of PC expression in pancreatic β islet cells, though no significant expression of PEPCK was observed (Bonner-Weir & Weir, 2004; Sugden et al., 2011). High fat diet mice also showed lower productions of malate, a gluconeogenic output, when treated with metformin (Lee et al., 2013). Reduction of malate following metformin treatment could suggest possible upstream inhibition of PC activity. PC loss of function in adipose and liver tissue leads to reduced fasting hyperglycemia, fasting plasma insulin concentrations, and endogenous glucose production in high-fat-fed rats (Kumashiro et al., 2013), its loss directly mimicking the antidiabetic effects of metformin. Overall, these studies suggest that PC may play a key role in the route by which metformin decreases hepatic gluconeogenesis and improves insulin secretion in type II diabetes, suggesting an unknown purpose for PC in these cells.

### Pharmacokinetics of Metformin

The maximum clinical dosage for metformin in humans is 35 mg /kg body weight (Ismail, Soliman, & Nassan, 2015). Following oral administration, metformin is absorbed into the gastrointestinal tract where it enters the liver via the hepatic portal vein (Figure 3). In the portal vein, plasma metformin concentrations are reported to be between 40-70 μM (Duong et al., 2013). Upon entry into the liver, metformin concentrations in primary hepatocytes rise to 220 μM (Jin et al., 2009). Following uptake from the liver, metformin is distributed into the rest of the body, where concentrations reportedly drop near a range of 10-40 μM (He & Wondisford, 2015).

*Figure 2.* The pathway for mitochondrial gluconeogenesis is enzyme coupled in nature. Pyruvate carboxylase catalyzes the first step and Malate Dehydrogenase the second. From "Gluconeogenesis for medical school," by R.,Kiran.http://www.slideshare.net /ravikiran35977897/gluconeogenesis-for-medical-school.

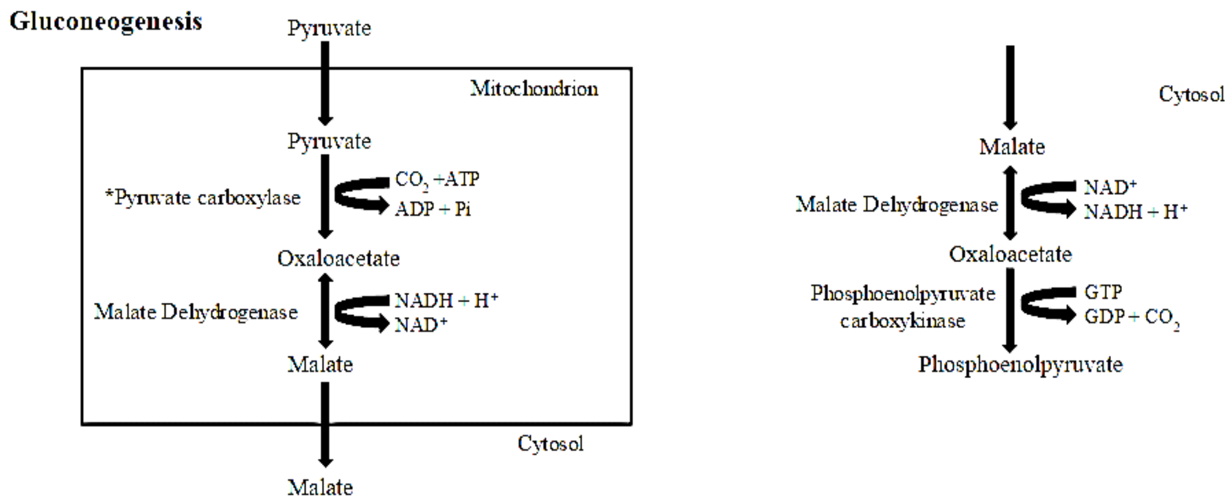
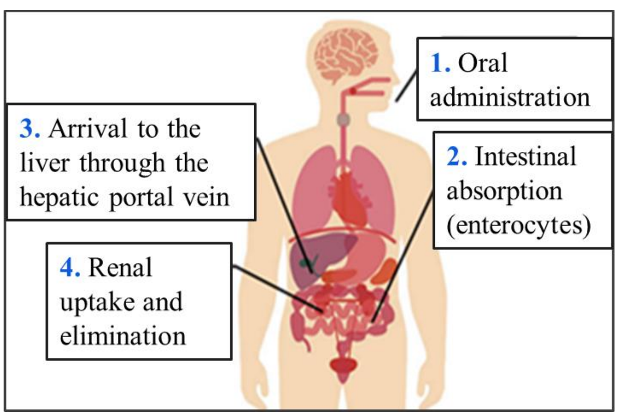



*Figure 3.* The pharmacokinetic route of metformin is illustrated. Once ingested, metformin is transported to the liver via the hepatic portal vein. Metformin proceeds to enter the general circulation system for renal absorption and elimination.




## The Adenosine Monophosphate-Activated Protein Kinase Pathway

Adenosine monophosphate activated protein kinase (AMPK) is an enzyme catalyzed pathway regulating cellular response, metabolism, growth and organelle function (Zaha & Young, 2012). The downstream LKB1 protein kinase is significant in the activation of the AMPK pathway during times of energetic stress (Foretz et al., 2005). Studies have claimed inhibition of gluconeogenesis by metformin actually results from the phosphorylation of the AMPK pathway (Cao et al., 2014; Shaw et al., 2005; Zhou et al., 2001). Non-obese mice without sufficient levels of hepatic LKB1 kinase demonstrated an impaired ability to reduce blood glucose levels following an intraperitoneal glucose injection (Shaw et al., 2005). In the same study, non-obese LKB1-deficient mice treated with metformin also failed to reduce blood glucose levels, while non-obese, wild-type mice demonstrate reduced levels by more than 50%. The LKB1 deficient mice were not obese when undergoing this study, a physical characteristic found in approximately 84.7% of adults, 18 or over, with the disease (Centers for Disease Control and Prevention, 2010).

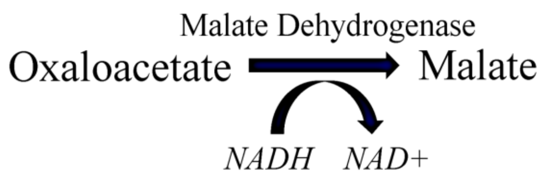
## METHOD

The hypothesis that metformin interacts in an inhibitory manner with PC action was assessed via kinetic analysis of the rate of the enzyme-coupled reaction of interest. Studies were performed through the Department of Chemistry and Biochemistry at the University of Northern Colorado, Greeley.

### Malate Dehydrogenase (MDH) Assay

To assure that metformin did not affect MDH activity, spectrophotometric assays (Figure 4) for MDH were run using metformin concentrations of 500μM, 1000μM and 5,000μM. All concentrations were prepared in ultra-pure water accordingly. Reaction media for all assays with and without metformin were mixed in a final 1mL cuvette volume with 0.20 mM NADH, 10 mM oxaloacetate, and MDH with 50 mM Tris-HCl buffer, pH 7.8, 30 ºC (Worthington Biochemical Corporation). Assays had a total run time of 1 min and a cycle time of 5 s.

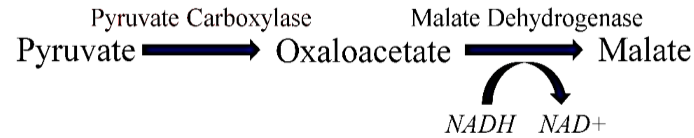
*Figure 4.* Reaction for MDH, part two of mitochondrial gluconeogenesis, is shown. Oxaloacetate is converted into malate via MDH using NADH as a coenzyme in the reaction. Loss of absorbance at 340 nm was used to determine the rate of the reaction.




### Pyruvate Carboxylase Assay

The protocol for the enzyme coupled reaction (Figure 5) was adapted from various sources (Bahl et al., 1997; Duggelby et al., 1982; Jitrapakdee et al., 2008; Warren & Tipton, 1974). Optimal assay temperature for the reaction was maintained at 30ºC with a total run time of 1 min and a cycle time of 5 s. Reaction media for all assays with and without metformin were mixed in a 1mL cuvette volume with Triethanolamine buffer, Magnesium Sulfate, Pyruvic acid and Bovine Serum Albumin (BSA) in buffer solution. MDH in excess was prepared in Aceytl-Coenzyme A solution and ATP were added to Triethanolamine (TEA) solution. PC enzyme solution contained Glycerol, Magnesium Acetate, and EDTA Tris-HCl buffer, adjusted to pH 7.8 using 1M HCl or 1M KOH as necessary, differing metformin solutions were prepared in ultra-pure water accordingly. For control trials, all conditions were kept constant and metformin volume was replaced by TEA (background) buffer during control trials.

*Figure 5.* The reaction for mitochondrial gluconeogenesis (above) were run for spectrophotometric analysis.




### Determination of Enzymatic Activity

A diode array spectrophotometer, provided by the University of Northern Colorado Department of Chemistry and Biochemistry, was utilized for kinetic measurements on the enzymatic reactions of interest (Figure 4 and 5). Using Beer's law, rates of the enzymatic reactions were converted into a rate, or loss of absorbance at 340 nm, in moles/sec. All runs were initiated with a blank test at an absorbance equal to 0 AU. The diode array spectrophotometer was allowed sufficient time (>30 min) for temperature equilibration prior to use.

### Statistical Analysis

Enzyme activity (rate = $\Delta \text{absorbance}^{340\text{nm}}/\text{sec}$) of experimental and control groups were compared analyzed via one-way analysis of variance (ANOVA) and significantly different groups were followed up by Waller-Duncan post-hoc analysis using SAS version 9.4 software (Cary, NC). What was your level of significance that you were looking for?

## RESULTS

### MDH and Metformin

Being that mitochondrial gluconeogenesis is enzyme-coupled in nature, it was necessary to assess potential inhibitory effects of metformin on MDH. One-way ANOVA revealed no significant difference between treated and controlled groups $F(3, 8) = 1.150$, $p = 0.38$ (Figure 7, Table 1).

### PC and Metformin

One-way ANOVA revealed a significant difference between treated and controlled groups, $F(4, 10) = 5.06$, $p = .0172$. The Waller-Duncan post-hoc analysis confirmed a significant difference between control and treated groups at 500µM, 1,000µM and 2,500µM ($M = 1.98\text{E} -7$). However, no significant difference for concentration group 250µM was observed compared to control (see Figure 8, Table 2).

*Figure 6.* The loss of absorbance at 340 nm is illustrated above. For every reaction run, the diode array provides similar outputs. The diagram serves to confirm the reaction has begun catalysis upon addition of ATP

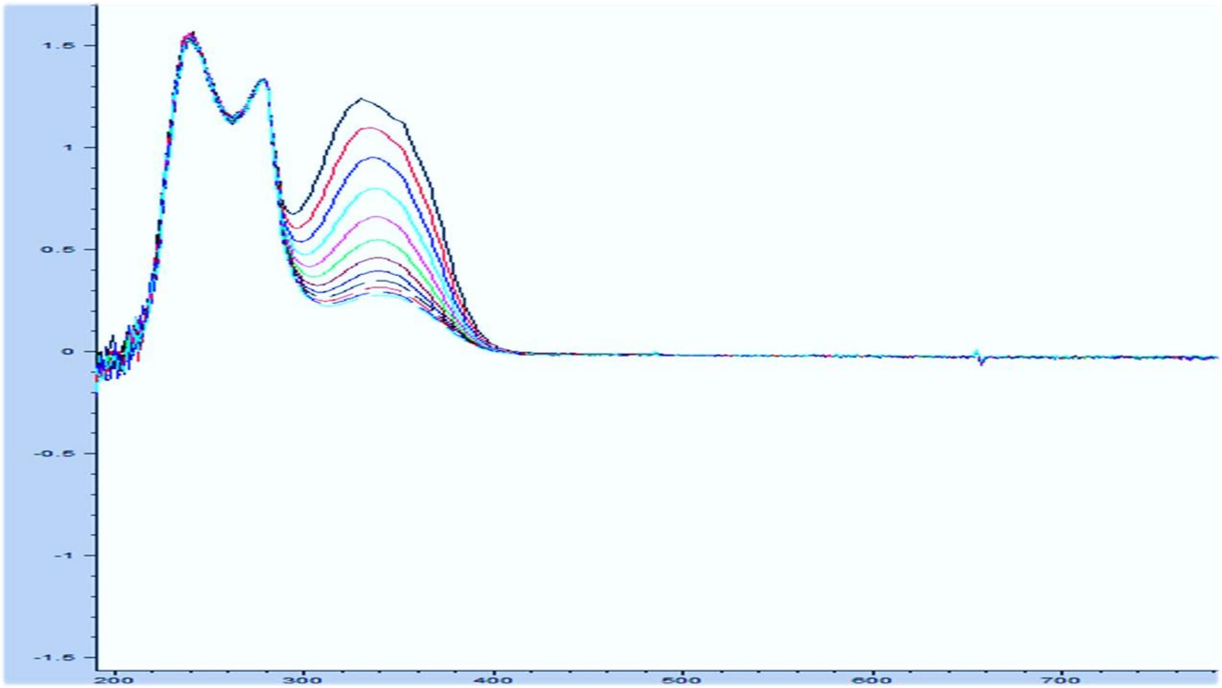



*Figure 7.* Graphical representation of the average rates (mol/sec) of MDH reactions treated with 500µM-5,000µM of metformin plus the controls are shown above. Rates have a downward slope signifying a loss of absorbance of NADH at 340nm. No significant differences were found between treated and controlled groups.

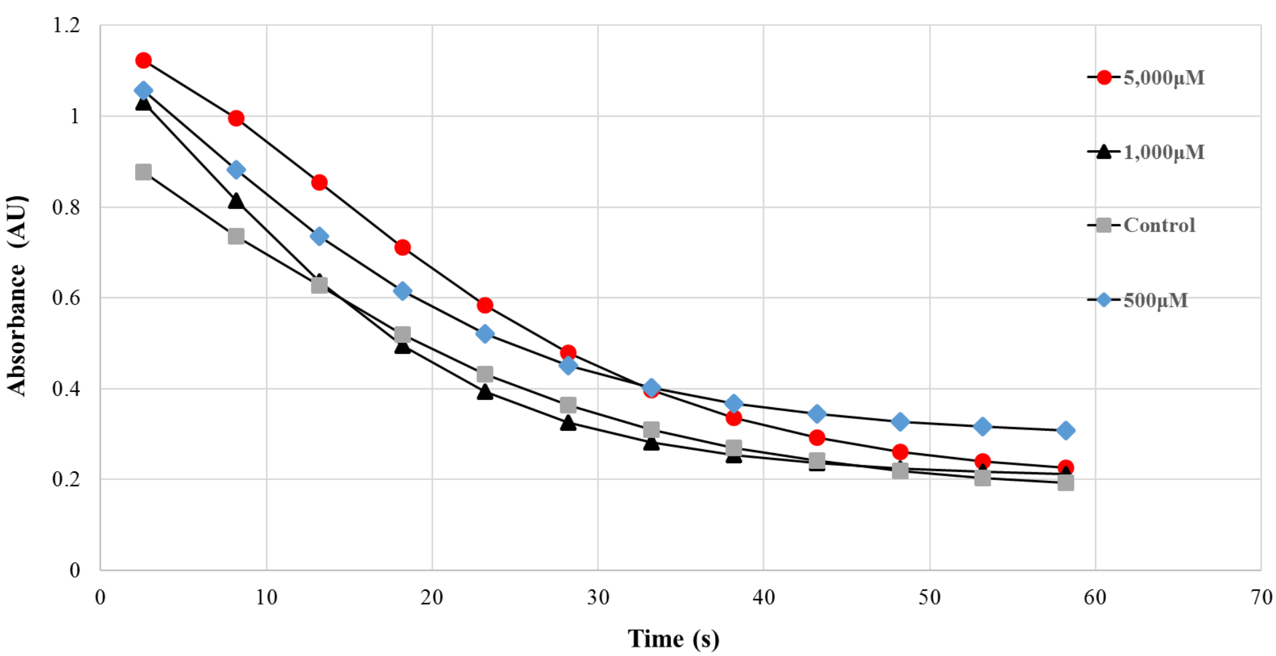


*Figure 8.* Graphical representation of the average rates (mol/sec) of the enzyme-coupled reactions treated with 250µM-2,500µM of metformin plus the controls are shown above. Significant differences between 500µM, 1,000µM and 2,500µM were found.

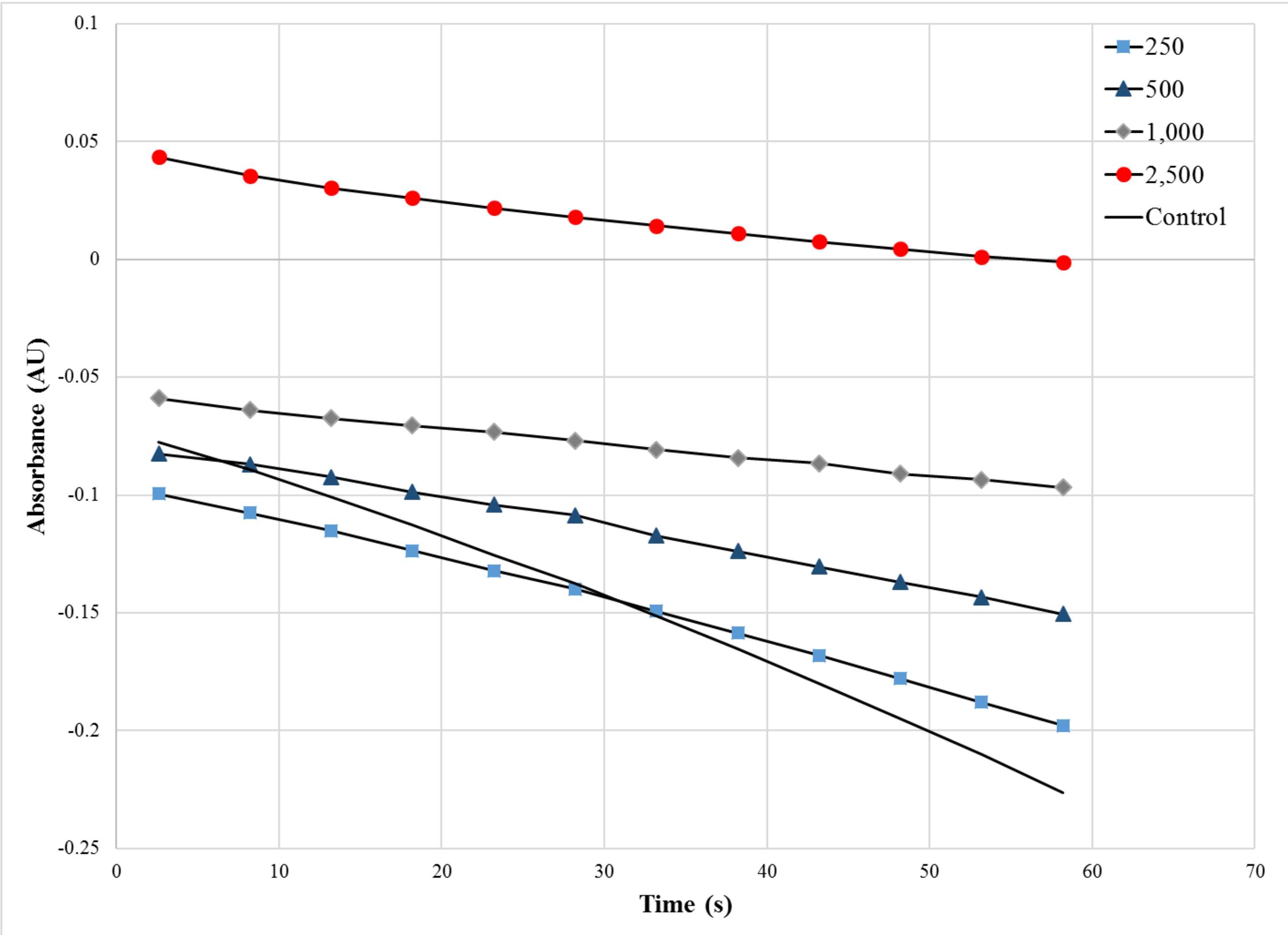


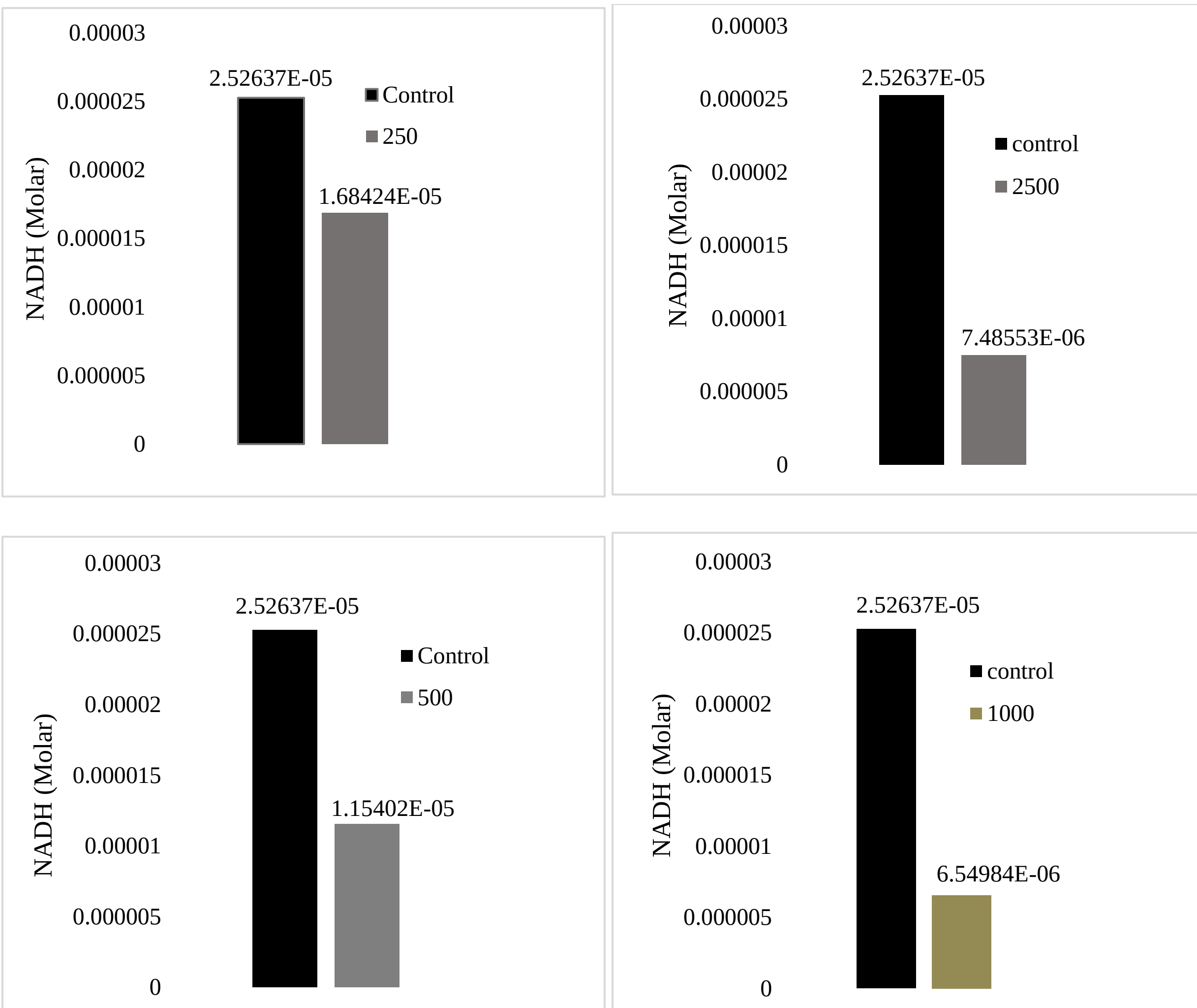

*Table 1.* Numerical data for average rates of MDH reactions run are provided above. Rates were not found to be statistically significant from control.

| Concentration | Avg. Rate (Mol/sec) |
|---|---|
| 500 | 2.04 E-6 |
| 1,000 | 1.75 E-7 |
| 5,000 | 2.83 E-7 |
| Control | 3.05 E-7 |

*Table 2.* Averages for the enzyme-coupled reaction are enumerated above. Significant differences* were found between 500-2,500µM and control.

| Concentration | Avg. Rate (Mol/sec) |
|---|---|
| 250 | 2.89 E-7 |
| 500 | 1.98 E-7* |
| 1,000 | 1.12 E-7* |
| 2,500 | 1.28 E-7* |
| Control | 4.34 E-7* |

*Figure 9.* Average NADH conversion to NAD+ per concentration of metformin.




## DISCUSSION

In this study, PC activity was not significantly inhibited for concentrations below 250µM. Duncan-Waller post-hoc analysis found that metformin treatment significantly inhibited pyruvate carboxylase activity at concentrations 500µM ≥ compared to control. As the concentration of metformin increased, the average rate of the reaction (Figure 8; Table 2). These results are consistent with those of other reported studies, as shown by Foretz et al (2010) and Cao et al (2014) where only concentrations above 500µM were found to significantly inhibit gluconeogenesis as a whole. On the contrary, this study focused solely on PC activity and not gluconeogenesis as a whole. Figure 9 illustrates the difference in conversion of NADH → $NAD^+$ between treated and controlled groups, where less conversion occurred as metformin concentrations increased, also supporting the observed results from this study.

Results from this study add to the present debate regarding metformin activity. Although concentrations above 1,000µM are not pharmacokinetically relevant to those reported in animals and humans (Duong et al., 2013; He and Wondisford, 2015; Jin et al., 2009), concentrations having a statistically significant inhibition on pyruvate carboxylase activity in this study were closer to physiological range than past studies, where metformin action was claimed to be mediated through the AMPK pathway using suprapharmacological concentrations around 10,000μM (He et al., 2009).

### Limitations

Due to small group sizes and the relative novelty of this study, difficulty arose when attempting to closely simulate potential encounters occurring *in-vivo.* A higher number of repetition will be needed to strengthen the study in the future.

### Future Studies

Results from this study are primary, as future research is needed to focus on examining the activity of PC at very specific concentration ranging between 70-250μM at high replication in order to observe exact thresholds at which metformin no longer inhibits PC activity. Assessment of this data may allow us to further judge the validity of these results, allowing us to uphold more concrete conclusions in the future. Future studies could also focus on assessing the potential interaction between metformin and other key gluconeogenic factors such as biotin, a prosthetic group of PC. $^{13}$C-NMR analysis may be used to assess the potential binding interaction through disappearance of key carbon groups of biotin, specifically the carbonyl side chain used to bind to PC.

### Implications

This study provides further observation that may help direct future studies regarding the mechanism of action for this drug and better understanding the mechanism. The importance of understanding the metformin route of action during type II diabetes is crucial. Metformin has become a drug of choice for these patients due to its low cost and relatively safe profile. Aside from being used to treat type II diabetes, metformin is also prescribed for weight loss in obese individuals (Inzucchi et al., 2012) and as a complimentary drug for PCOS patients to induce ovulation. The drug has recently been shown to contain anti-cancer properties by increasing survival rates in patients with breast cancer (University of Pennsylvania School of Medicine, 2016). Metformin continues to be the most popular around the world among other glucose lowering agents such as thiazolidinediones, sulfonylureas and others. Being that metformin is in high demand, it is important to keep characterizing the symptoms, side effects and benefits along with the mechanism behind them.

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